AGGLUTINATION OF FISH AND TURTLE ERYTHROCYTES BY VIRUSES[1]

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Following the observation by Hirst (1941) that influenza viruses agglutinate chicken erythrocytes, it was found that several other viruses are capable of agglutinating erythrocytes from certain animal species (Burnet, 1942; Lush, 1943; Levens and Enders, 1945; Nagler, 1942; Burnet and Boake, 1946; Mills and Dochez, 1944; Hallauer, 1949; Lahelle and Horsfall, 1949; Olitsky and Yager, 1949; Sabin and Buescher, 1950). Moreover, it was demonstrated that anti- or immune serum against the corresponding virus inhibits hemagglutination by the agent. As a result, the hemagglutination reaction became a much used technique for measuring either viral or antibody concentration and is useful also in the serologic diagnosis of a number of viral diseases. The reaction of influenza, mumps, and Newcastle disease viruses with erythrocytes proved of special interest when it became evident that these viruses are able to elute spontaneously from erythrocytes with which they combine (Hirst, 1942). As the virus elutes, the erythrocytes lose their capacity to adsorb fresh virus. This loss is accompanied by a reduction in the electrophoretic mobility of the erythrocytes (Hanig, 1948; Ada and Stone, 1950; Stone and Ada, 1950). The eluted virus, however, is capable of combining with and agglutinating fresh erythrocytes (Hirst, 1942). A considerable amount of evidence has accumulated favoring the view that influenza, mumps, and Newcastle disease viruses are capable of effecting an enzymatic alteration of receptor areas on the erythrocyte surface. It appears probable that the receptors are mucoprotein in nature. It should be emphasized that none of these viruses is capable of multiplying in erythrocytes. What role such enzymatic activity has in the series of events leading from virus-host cell combination to multiplication of viruses in and their subsequent release from host cells is not established despite interesting work bearing on this problem (Stone, 1948a; 1948b).

The present study was undertaken in order to extend the range of observations in respect to erythrocyte-virus interaction. Erythrocytes from certain lower classes of vertebrates were employed. In addition to viruses known to cause agglutination of erythrocytes from higher classes of vertebrates, other viruses which have not been reported to agglutinate erythrocytes were studied.

Materials and Methods

Viruses. Ten-day old embryonated chicken eggs were inoculated allantoically with PR8 or FM1 strain of influenza A virus, Lee or MB strain of influenza B virus, swine influenza, or Newcastle disease virus. The eggs were incubated at 35° C. for

[1] Part of the work was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

48 hours, then chilled at 4° C. overnight, and the allantoic fluid harvested. Habel or Enders strain of mumps virus was inoculated in 7-day old chicken embryos and allantoic fluid harvested 5 days later. The allantoic fluids were dialyzed against 80 volumes of 0.1 *M* phosphate buffer at pH 7.1, centrifuged at 3800 g for 10 minutes and the supernatants stored at 4° C. In addition, Lee virus was dialyzed against 0.85 per cent NaCl. Herpes simplex virus, K strain, kindly made available by Dr. E. D. Kilbourne, was inoculated on the chorio-allantoic membrane of 10-day old chick embryos. The membranes were harvested after incubation of the eggs at 35° C. for two days. A 50 per cent suspension was then prepared and homogenized in a modified Waring blendor. The suspension was clarified by centrifugation at 3800 g for 10 minutes and the supernatant stored at 4° C. Semliki Forest virus was inoculated allantoically in 10-day old chick embryos. The eggs were incubated for 30 hours. Allantoic fluid was harvested, dialyzed, centrifuged and stored as described above. The infected embryos, too, were collected and a 50 per cent suspension prepared and stored as described above. Columbia MM strain of mouse encephalomyelitis virus was inoculated intracerebrally in 5-week old white mice. The mice were killed two days later and a 25 per cent suspension of infected brain was prepared as described above. Coxsackie virus (Conn. No. 5 strain) was inoculated in one-day old suckling mice. The mice were killed four days later. A 10 per cent suspension of the carcasses was prepared, homogenized and centrifuged at 3800 g for 30 minutes. The supernatants were stored at 4° C. Pneumonia virus of mice (PVM) was inoculated intranasally in three-week old mice. The mice were killed 6 days later and the lungs collected. A 10 per cent suspension in water was prepared, homogenized, heated at 70° C. for 30 minutes, and clarified by centrifugation at 3800 g for 10 minutes. The supernatant was collected and stored at 4° C. Uninfected control suspensions of each of the various host tissues used were prepared.

In experiments with erythrocytes, untreated viruses or viruses heated at 56° C. for 30 minutes were employed as described below.

Erythrocytes. Blood, obtained with syringe and needle, was mixed with ⅙ volume of a 2.5 per cent solution of sodium citrate. Two or more representatives of each species of animals were bled and their RBC tested. Erythrocytes were washed three times with appropriate diluent (see below) and a 1 per cent suspension prepared in the same diluent. It was found that erythrocytes from dogfish and turtle sedimented more rapidly in the centrifuge than those from mackerel, sea robin or tautog.

Dogfish (*Mustelus canis*). Blood was obtained from the caudal vein. The freezing point depression of blood of marine elasmobranchii is − 1.93 (Smith, 1931) which corresponds to the freezing point depression given by a 3.26 per cent solution of NaCl (Roth and Scheel, 1923). Because of the high concentration of urea in the blood, the diluent chosen for dogfish erythrocytes contained 1.64 per cent NaCl and 2.16 per cent urea (Lutz, 1930). Other salts were omitted.

Mackerel (*Scomber scombrus*), *sea robin* (*Prionotus carolinus*), *tautog* (*Tautogo onitis*). Blood was obtained by cardiac puncture. The freezing point depression of blood of typical marine teleosts is − 0.8 (Smith, 1931). Correspondingly, a 1.37 per cent solution of NaCl was used as the suspending agent (Roth and Scheel, 1923).

Turtle (*Pseudemys elegans*). Blood was obtained by cardiac puncture through a drill hole in the plastron. On the basis of freezing point depressions reported for

various turtles (Botazzi, 1908; Burian, 1910), a 0.86 per cent solution of NaCl was used as the medium for turtle erythrocytes.

Hemagglutination tests. Serial twofold or tenfold dilutions of virus were prepared in 0.4 cc. volumes in the appropriate diluent and 0.4 cc. of 1 per cent erythrocytes added per tube (final concentration 0.5 per cent). The tubes were maintained at 25 or 5° C. for periods of time varying from 10 to 80 minutes, depending on speed of sedimentation and disagglutination of erythrocytes, as indicated below. The tests then were read. The last tube showing definite (2 + to 3 +) agglutination was taken as the end point. Titers are expressed as the reciprocal of the dilution of virus at the end point.

Experimental

Agglutination of fish and turtle erythrocytes by viruses. In the initial experiments with erythrocytes and viruses employed in this study, hemagglutination tests were carried out at 25 and 5° C. with three final concentrations of virus representing

Table I

Agglutination of marine erythrocytes by influenza, mumps and Newcastle disease viruses

Erythrocytes	Virus							
	Influenza A		Influenza B		Swine influenza	Mumps		Newcastle disease
	PR8	FM1	Lee	MB		Enders	Habel	
Dogfish	+*	+	+	+	+	+	+	+
Sea robin	+	+	+	+	+	+	+	+
Mackerel	−†	−	−	+	+	+	+	+
Tautog	+	+	+	+	+	+	+	+
Turtle	−	−	−	+	+	+	+	+

* Plus sign indicates that definite hemagglutination was present at a dilution of 1:20 or higher.
† Minus sign indicates that no evidence of hemagglutination was present at a dilution of 1:2. Because qualitatively identical results were obtained at 25 and 5° C., each sign represents results recorded at both temperatures.

1 : 2, 1 : 20 and 1 : 200 diluted allantoic fluid, mouse lung or mouse brain suspension. Table I summarizes the results of these experiments with various strains of influenza, mumps and Newcastle disease viruses. As can be seen, hemagglutination was observed in the great majority of tests. However, striking specificity was shown with PR8, FM1 and Lee strains of influenza virus which failed to agglutinate mackerel and tautog erythrocytes either at 25 or 5° C.

No agglutination of dogfish, mackerel, sea robin, tautog or turtle erythrocytes was observed under identical conditions with the following viruses: herpes simplex, Semliki Forest, Columbia MM strain of mouse encephalomyelitis, Coxsackie (Conn. No. 5) and pneumonia virus of mice (PVM).

Control experiments with normal allantoic fluid, suspensions of normal mouse lung and mouse brain gave no evidence of agglutination.

Comparison of hemagglutination titers. Lee and MB strains of influenza B virus were employed in comparative experiments with various erythrocytes. It was de-

sired to determine the range of hemagglutination titers at two temperatures: 25 and 5° C. In addition, hemagglutination titers with viruses heated at 56° C. for 30 minutes were measured.

Table II gives the results of representative experiments of this kind. As can be seen, the hemagglutination titers at 25° C. fall within a range of 256 to 4026, with the exception of low titers obtained with Lee virus when sea robin erythrocytes were used and with MB virus and mackerel red blood cells. In the latter instances the titers observed at 60 minutes were lower than those at 30 minutes in contrast to the stable values observed with other virus-erythrocyte combinations within the time interval indicated.

The hemagglutination titers obtained at 5° C. were within the same high range as, or in certain instances somewhat higher than, the titers obtained at 25° C. In

TABLE II

Agglutination of marine erythrocytes by Lee and MB strains of influenza B virus

Treatment of virus	Temp. of agglut.	Lee virus						MB virus				
		Reading	Dog-fish	Mack-erel	Sea robin	Tau-tog	Turtle	Dog-fish	Mack-erel	Sea robin	Tau-tog	Turtle
	° C.											
None	25	1st*	1,024	0	16	1,024	0	2,048	64	256	512	256
		2nd†	1,024	0	4	1,024	0	4,096	8	256	512	256
56° C., 30′	25	1st	1,024	0	64	1,024	0	4,096	128	128	256	128
		2nd	1,024	0	64	1,024	0	4,096	128	128	256	256
None	5	1st	2,048	0	512	1,024	0	4,096	256	512	512	512
		2nd	2,048	0	512	1,024	0	4,096	256	512	512	512

* 1st reading at 20 minutes with dogfish and turtle erythrocytes, and at 30 minutes with mackerel, sea robin and tautog erythrocytes.

† 2nd reading at 40 minutes with dogfish and turtle erythrocytes, and at 60 minutes with mackerel, sea robin and tautog erythrocytes.

particular, Lee virus and sea robin erythrocytes as well as MB virus and mackerel erythrocytes gave high titers at this temperature.

Hemagglutination titers obtained at 25° C. with heated viruses (56° C., 30 minutes) were, in general, closely similar to those observed with unheated agents at this temperature. However, heated Lee virus and sea robin erythrocytes, as well as heated MB virus and mackerel erythrocytes, gave titers somewhat higher than those observed with unheated viruses at this temperature. Moreover, the titers observed were stable, in contrast to those obtained with untreated viruses at this temperature. It should be noted that the titers observed with these two viruses (untreated) at 5° C. were still higher.

Disagglutination of erythrocytes. In view of the evidence that elution of influenza viruses from erythrocytes is accompanied by loss of the ability of such erythrocytes to agglutinate with the same virus (Hirst, 1942), experiments were done to determine whether elution of the virus and disagglutination or dispersion of agglutinated erythrocytes were correlated in time.

The Lee and MB strains of influenza B virus employed were dialyzed against 0.85 per cent NaCl and 0.1 *M* phosphate buffer, respectively. Chicken erythrocytes washed with and suspended in 0.85 per cent NaCl were used. Preliminary experiments were done to determine the most suitable proportions of virus and erythrocytes. If too high relative concentrations of virus are used, the considerable amounts of uncombined virus in the system interfere with measurement of the quantities of virus adsorbed or eluted per unit of time. On the other hand, if too high relative concentrations of erythrocytes are used, the large number of unagglutinated erythrocytes interferes with the counting of disagglutinated cells. These experiments showed that satisfactory adsorption-elution and agglutination-disagglutination curves could be obtained when undiluted allantoic fluid (with a hemagglutination titer of 1024) was mixed with an equal volume of 10 per cent erythrocytes.

Preliminary experiments were also done to determine the optimal temperature for elution, and with Lee virus 14° C. was chosen, whereas with MB virus elution curves were determined at 37° C. This difference indicates that Lee virus has a much faster rate of elution from chicken erythroyctes than MB virus.

The adsorption-agglutination and elution-disagglutination experiments were done as follows: Virus preparation and erythrocyte suspension were mixed at 4° C. in a 25 × 110 mm. test tube with a side arm. Homogeneous distribution of agglutinated and unagglutinated erythrocytes in the mixture was maintained by mechanical stirring at a fixed rate. At intervals, aliquots were removed with a pipette via the side arm. The erythrocytes in a part of each aliquot were promptly sedimented by centrifugation. The supernatant was collected and the concentration of virus determined by means of hemagglutination titration. The other portion of the aliquot was diluted 1 : 5 with 0.85 per cent NaCl and unagglutinated erythrocytes were counted in a hemocytometer. After a suitable period of time, the reaction mixture was brought to the temperature at which elution-disagglutination could be followed optimally and additional aliquots were removed and examined as described above.

Figure 1 gives the results of representative experiments with Lee and MB viruses. As can be seen, elution of virus and disagglutination of erythrocytes followed essentially parallel courses.

In experiments with erythrocytes from marine species, a large number of instances of spontaneous disagglutination of erythrocytes was observed. The results with Lee virus and sea robin erythrocytes and with MB virus and mackerel erythrocytes were discussed above. In addition, similar evidence was obtained with dogfish, tautog and turtle erythrocytes. These experiments were carried out at 25° C. The period of observation was prolonged to four hours by re-suspension of sedimented erythrocytes by shaking at regular intervals. Disagglutination of erythrocytes from each of the five species employed was observed with two or more influenza virus strains and with Newcastle disease virus. Agglutination of erythrocytes by mumps virus was followed by spontaneous disagglutination in the case of turtle, mackerel and dogfish erythrocytes.

In different virus-erythrocyte systems, loss of agglutinability appeared after strikingly different periods of time. The rate of action of Newcastle disease and Lee viruses on dogfish and sea robin erythrocytes was found to be considerably faster than that of other viruses employed, including PR8, FM1, MB, swine and mumps. Moreover, marked differences were noted in respect to the time required by different erythrocytes to lose their agglutinability when mixed with one and the same virus,

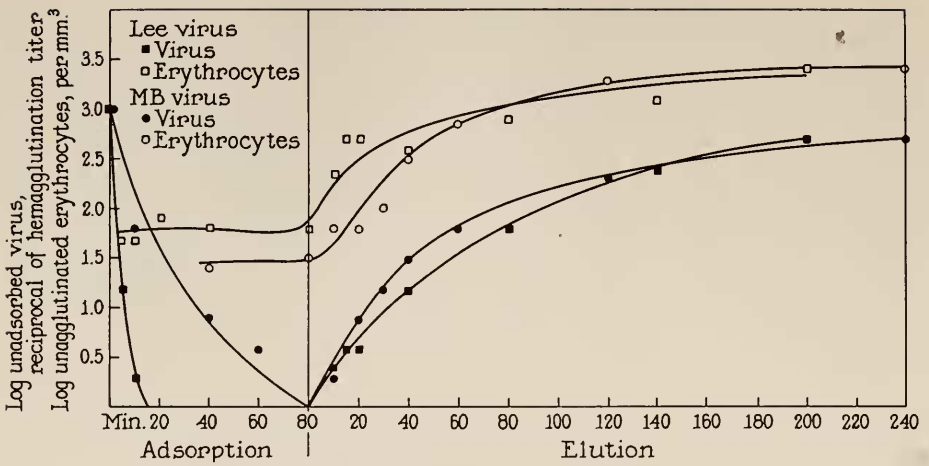

FIGURE 1. Relation of adsorption and elution of virus to agglutination and disagglutination of erythrocytes. With both Lee and MB viruses, adsorption was carried out at 4° C. Elution was permitted to take place at 14° C. with Lee virus, and at 37° C. with MB virus. In both experiments the control virus titer was 1024. Chicken erythrocytes, final concentration 5 per cent, were used.

e.g., employing MB virus, longer periods were required with tautog, sea robin and dogfish erythrocytes than with mackerel or turtle cells. In no case was evidence of disagglutination observed when tests were done at 4° C.

It appeared, therefore, that disagglutination was a regular phenomenon and that the exceptional instances where disagglutination was not observed may have been due to experimental conditions used: if temperatures higher than 25° C. and periods longer than 4 hours had been employed, disagglutination might have occurred in such instances.

DISCUSSION

The finding that influenza, mumps and Newcastle disease viruses are capable of agglutinating erythrocytes from teleosts and elasmobranchii is of interest because it focuses attention on the extremely broad species range of this reaction: the agglutination of reptilian, amphibian, avian and mammalian erythrocytes by representatives of this group of viruses has been demonstrated previously (Smadel, 1948). Studies with chicken erythrocytes have yielded suggestive evidence that in the hemagglutination reaction influenza, mumps and Newcastle disease viruses combine with receptor groups on erythrocytes which are mucoprotein in nature (de Burgh *et al.*, 1948; Hirst, 1948). Strong, although indirect, support for this contention has come from studies with mucoid components of tissues and body fluids which react with influenza, mumps and Newcastle disease viruses (Tamm and Horsfall, 1952). One of these components, a mucoprotein present in normal human urine, has been shown, following isolation and purification, to be a single homogeneous substance by electrophoretic and ultracentrifugal criteria (Perlmann *et al.*, 1952; Bugher, 1951). The features which characterize virus-erythrocyte interaction also characterize that between mucoprotein from urine and influenza, mumps or Newcastle disease viruses:

combination between the reactants is followed by emergence of free virus and demonstrable alteration in the mucoprotein. The altered mucoprotein is not able to combine with virus and its electrophoretic mobility is reduced (Perlmann *et al.*, 1952). As in the case of virus-erythrocyte interaction, the rate at which the virus causes alteration of the mucoprotein is considerably faster with certain viruses than with others. For example, the rate of Lee or Newcastle disease virus action on the mucoprotein is more rapid than that of MB, FM1, mumps or swine influenza viruses (Tamm and Horsfall, 1952). The findings reported in this study are in agreement with these observations.

Thus, it appears probable that erythrocytes from species ranging from man to the dogfish possess similar surface receptor areas with which influenza, mumps or Newcastle disease viruses are able to combine, and which they alter during the process of elution. However, within the species range of virus-erythrocyte interaction, instances can be found in which agglutination does not take place, as illustrated by the failure of PR8, FM1 or Lee viruses to agglutinate mackerel or turtle erythrocytes. Structural fit as well as adsorptive forces may be factors involved in this phenomenon for which at present an explanation is lacking.

In contrast to the group of viruses discussed above, pneumonia virus of mice, the various mouse encephalomyelitis viruses, and Japanese B encephalitis virus are agents which agglutinate erythrocytes from only a very few animal species. Agglutination of erythrocytes by these viruses takes place under conditions which are different in each case. These agents do not show spontaneous elution and nothing definite is known about the chemical nature of the groupings on the red cell surface with which these viruses combine. It should be emphasized that numerous viruses have failed to give the hemagglutination reaction with any erythrocytes against which they were tested.

Summary

1. Dogfish, sea robin or tautog erythrocytes are agglutinable by all strains of influenza, mumps or Newcastle disease viruses employed.

2. Mackerel and turtle erythrocytes are agglutinable by mumps or Newcastle disease viruses as well as by some strains of influenza B virus. In the presence of these agents, agglutinable erythrocytes lose their capacity to be agglutinated by the same virus and spontaneous elution of the agent occurs. Elution of virus from erythrocytes and disagglutination appear to occur concurrently.

3. Among a number of other viruses tested, none was capable of agglutinating erythrocytes employed in this study.

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